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Widespread occurrence of the micro-organism *Wolbachia* in ants

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For more than 20 years, sex allocation in hymenopteran societies has been a major topic in insect sociobiology. A recurring idea was that relatedness asymmetries arising from their haplodiploid sex determination system would lead to various parent–offspring conflicts over optimal reproduction. A possible weakness of existing theory is that only interests of nuclear genes are properly accounted for. Yet, a diversity of maternally transmitted elements manipulate the reproduction of their host in many solitary arthropod groups. The bacterium *Wolbachia* is a striking example of such a selfish cytoplasmic element, with effects ranging from reproductive incompatibility between host strains, induction of parthenogenesis and feminization of males. This paper reports on a first PCR-based *Wolbachia* screening in ants. Out of 50 Indo-Australian species, 50% screened positive for an A-group strain. One of these species also harboured a B-group strain in a double infection. Various factors that might explain the unusually high incidence of *Wolbachia* in ants are discussed. In general, *Wolbachia* may represent a widespread and previously unrecognized party active in the conflicts of interest within social insect colonies.

Keywords: *Wolbachia*; *ftsZ*; ants; selfish cytoplasmic elements

1. INTRODUCTION

After more than 20 years, the seminal papers by Hamilton (1964*a*,*b*) and Trivers & Hare (1976) have sparked off an enormous number of studies dealing with sex ratio evolution and reproductive conflicts in hymenopteran societies (Bourke & Franks 1995; Crozier & Pamilo 1996). A recurring idea was that their haplodiploid sex determination system makes full-sisters exceptionally highly related, because of the shared genes contributed by their haploid father (Hamilton 1964*b*). Moreover, in colonies headed by a single, once-mated queen, the non-reproductive workers would pass on copies of their own genes three times as efficiently through female full-sister reproductives (related by 3/4) than through brother reproductives (related by 1/4), or 50% more efficiently through their own sons (mostly full-nephews, related by 3/8) than through queen's sons (brothers, related by 1/4). This unusual relatedness setting would lead to various conflicts between the queen and her worker offspring over optimal reproduction, e.g. regarding male parentage and optimal sex allocation (Trivers & Hare 1976).

A possible weakness of the current theories on reproductive conflicts in hymenopteran societies is that only interests of nuclear genes are properly accounted for. Yet, in many solitary arthropod groups, a diversity of maternally transmitted elements manipulate their host's reproduction to their own advantage thereby opposing the interests of the nuclear genes (Hurst 1993; Hurst *et al.* 1997). Most of these elements cause their host to produce broods of mostly female offspring, the sex which maximizes their spread owing to the exclusive maternal inheritance, and have been termed cytoplasmic sex ratio distorters. Strategies that cause female bias range from male killing (reviewed by Hurst (1991)), induction of all-female parthenogenetic reproduction, feminization of genetic males and increasing the fertilization frequency in male haploids ('maternal sex ratio'; Skinner 1982).

The maternally inherited bacterium *Wolbachia* is a striking example of such a selfish cytoplasmic element, with effects ranging from reproductive incompatibility between host strains ('cytoplasmic incompatibility'; Barr 1980; O'Neill & Karr 1990; Breeuwer & Werren 1990; reviewed by Hoffmann & Turelli (1997)), parthenogenesis induction in some parasitoid wasps (Stouthamer *et al.* 1993; Schilthuizen & Stouthamer 1997; reviewed by Stouthamer (1997)) and feminization of genetic males in isopods (Rousset *et al.* 1992; Martin *et al.* 1994; reviewed by Rigaud *et al.* (1997)). Recent surveys have found both of the major strains of *Wolbachia* (designated A and B; Rousset *et al.* 1992; Breeuwer *et al.* 1992; Stouthamer *et al.* 1993; Werren *et al.* 1995*a*) in over 16% of New World insect species across all major orders (Werren *et al.* 1995*b*). In addition, the bacteria have been found in isopods (Rousset *et al.* 1992) and in mites (Johanowicz & Hoy 1995; Tsagkarakou *et al.* 1996; Breeuwer & Jacobs 1996), and a close relative has been reported in a nematode (Sironi *et al.* 1995). Cytoplasmic incompatibility seems to be the most common mode of action, involving a kin-selected (Frank 1997) reproductive incompatibility between infected sperm and uninfected eggs, resulting in zygotic death in diploid species (Yen & Barr 1971) or male production in haplodiploids (Saul 1961; Ryan &

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Saul 1968; Breeuwer & Werren 1990). A general review of the biology of *Wolbachia* can be found in Werren (1997) and O'Neill *et al.* (1997).

Several authors have suggested that *Wolbachia* might also influence reproductive patterns in social Hymenoptera (Crozier & Pamilo 1993, 1996; Bourke & Franks 1995; Heinze & Tsuji 1996). Various factors add up to the intricate complexity of selfish cytoplasmic elements in social species, calling for a *Wolbachia* screening in this group. In particular, an incompatibility micro-organism is expected to spread more easily in a social species compared with a solitary one because effects of cytoplasmic incompatibility would be expressed both at the individual and 'society' levels. The aim of the present paper is to report on a first detailed *Wolbachia* screening in ants, generally regarded as model organisms for the study of social organization. We focus on the tropical Indo-Australian ant fauna, where all major ant subfamilies coexist and species of various sociogenetic structures are found. All species are surveyed for *Wolbachia* using polymerase chain reaction (PCR) amplification of the bacterial *ftsZ* protein-coding gene. Various factors that might influence the invasion and/or maintenance of *Wolbachia* in social Hymenoptera are discussed.

2. MATERIAL AND METHODS

(a) *Sampling and DNA extraction*

Workers of 50 ant species were collected in the national reserves of Kebun Raya Bogor, Ujung Kulon, Mt Gede, Mt Sarak-Bogor (West Java) and Padang (West Sumatra) during April 1997 and were subsequently preserved in 95% ethanol. In the light of previous results (T. Wenseleers, unpublished data) that have shown near absence of any intrapopulation infection polymorphism and equally efficient detection of *Wolbachia* in workers, males and alate queens, only workers of a single colony per species were included in the present study. The pre-extraction treatment and all DNA extraction procedures were performed under a laminar flow hood in a separate laboratory facility that is not located in proximity to the laboratory where PCR amplification or DNA sequencing took place. Possible external contamination was eliminated by brief immersion in 70% EtOH, followed by two rinses of double-distilled water and exposure for 5 min to 250 nm UV. DNA was extracted by boiling the pooled contents of the abdomens minus the digestive tracts (to eliminate the risk of contamination from infected prey species (Johanowicz & Hoy 1996)) of six workers in 300 µl (600 µl for large species) of a 10% Biorad Chelex 100 resin solution for 30 min. Because six individuals of each species were used, and assuming complete absence of any experimental failure in DNA extraction or amplification, one can calculate from the binomial distribution that a power of *ca.* 90% was attained to detect *Wolbachia* if it were present in only 20% of the workers. The samples were centrifuged and stored at −20 °C before use. Samples of all species investigated in this study have been deposited in the collection of the Bogor National Reserve for future taxonomic reference.

(b) *PCR amplification*

Based on GenBank-deposited *Wolbachia ftsZ* sequences (Holden *et al.* 1993; Werren *et al.* 1995*a*; Hoshizaki & Shimada 1995), primer pairs were developed which selectively amplify A- and B-group *Wolbachia*. The general forward primer FtsZFT2 5′-GAAGGTGTGCGACGTATGCG-3′ combined with the specific reverse primers FtsZRTA1 5′-CTCTGAGTCTTCGCT-CTGACTTATAGG-3′ and FtsZRTB2 5′-ACTCTTTCGTTTG-TTTGCTCAGTTG-3′ were used to amplify a 591-base pair (bp) and 670-bp stretch of the *Wolbachia ftsZ* gene of strains A and B, respectively. Results have shown that these primer pairs are specific for *Wolbachia*, as they fail to amplify other members of the alpha proteobacteria such as the closely related rickettsial *Adalia bipunctata* bacterium (Stouthamer *et al.* 1993), and the more distantly related *Agrobacterium tumefaciens*, *Bartonella bacilliformis*, *Caulobacter crescentus* and *Sinorhizobium meliloti*. The following insect mitochondrial DNA (*COII* gene) specific primers were used to ascertain the quality of all DNA samples used: CII 1-1 5′-CTTTATCAACATTTATTTTGATTTTTT-3′ and CII 1-2 5′-TACTCCAATAAATATTATAATAAATTG-3′ (for more details see Hoshizaki & Shimada (1995)).

The PCR amplification reactions were carried out in 15 µl reaction mixtures consisting of 0.5 µM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 5 µl of the crude DNA extract, 0.3 U of Taq polymerase (AmpliTaq, Perkin Elmer Cetus) and 1 × enzyme buffer supplied by the manufacturer. Each reaction mixture was overlaid with about 20 µl of mineral oil. PCR was performed with initial denaturation at 94 °C for 5 min, followed by 45 cycles consisting of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min, and a final 10 min extension at 72 °C. A sample of 10 µl of this reaction mixture was electrophoresed with a 100 bp DNA ladder size standard (GibcoBRL) on 1.5% agarose minigels. DNA bands were visualized by ethidium bromide staining. Two *Nasonia vitripennis* strains (LbII, harbouring *Wolbachia* A- and B- group clones, and TX, uninfected) were included as positive and negative controls in every amplification.

3. RESULTS

Out of a total of 50 ant species screened, 25 (50%) proved to be infected by an A-group *Wolbachia* (table 1). Only one *Monomorium* species carried a B-group strain in a double infection. Seven out of eight subfamilies surveyed contained infected species (for the Dorylinae, only one species was censused, which turned out to be negative), and there was no significant subfamily-related bias in susceptibility ($\chi^2 = 3.7$, d.f. $= 7$, $p > 0.1$).

Looking at table 1 for possible patterns in infection status, one can see that there is a weak association between *Wolbachia* prevalence and mode of colony foundation. In ants, colonies propagate either by queens unassisted by workers (independent founding, 'haplometrosis'), by a foundress association of several cooperating queens (dependent-founding, 'pleometrosis'), or by queens assisted by workers (dependent founding). In the latter case, colonies either split up into a number of daughter colonies, each headed by a new queen, immediately after sexuals have been produced (dependent founding, fissioning), or they release mated queens accompanied by buds of workers (dependent founding, budding). Out of eight obligate haplometrotic species (*Polyrhachis cryptoceroides*, *Pheidole* sp. 1, *P. tandjongensis*, *Pristomyrmex brevispinosus*, *Gnamptogenys costata*, *Hypoponera* sp., *Odontomachus simillimus* and *Probolomyrmex dammermani*), only *Probolomyrmex dammermani* was *Wolbachia*-positive (13%) versus 11 out of 22 (50%) for (facultatively) dependent-founding species. This difference is on the borderline of statistical significance ($\chi^2 = 0.06$, d.f. $= 1$, $p = 0.06$). In addition, most

Table 1. *Ant species surveyed for* Wolbachia *infection*

(Except when noted, all material was collected in the Kebun Raya Bogor National Reserve of West Java. Social structure (M, monogyny; P, polygyny), type of reproduction (performed by AQ, alate queens; EQ, wingless ('ergatoid') queens; G, mated workers ('gamergates'); GQ, gamergates and/or queens), mode of colony propagation (H, independent ('haplometrosis'); P, foundress associations ('pleometrosis'); B, budding; F, fissioning) and nesting habits (A, arboreal; S, soil nesting) are shown whenever data could be recorded. Species for which these characters could be measured accurately are asterisked and have been included in the statistical treatment.)

subfamily species (Bogor National Reserve Collection access number)	*Wolbachia* strain	social structure (M/P)	type of reproduction (G/GQ/EQ/AQ)	colony propagation (H/P/B/F)	nesting habits (A/S)
Formicinae	6/8=75%				
Acropyga activentris	A	M?	AQ?	H?	S
**Anoplolepis longipes*	A	P	AQ	H/B	S
Paratrechina sp.1 (FI97-5)	A	?	AQ?	?	S
Paratrechina sp. 2 (FI97-17)	A	?	AQ?	?	S
**Polyrhachis cryptoceroides*	—	M	AQ	H	A
Polyrhachis sp. (FI97-623)	—	?	AQ	?	A
Pseudolasius sp. 1 (FI97-77)	A	?	AQ	?	S
Pseudolasius sp. 2 (FI97-625)	A	?	AQ	?	S
Dolichoderinae	2/4=50%				
Dolichoderus sulcaticeps	—	?	AQ	?	A
* *Dolichoderus thoracicus*	—	P	AQ	H/B	A
**Tapinoma* sp. (FI97-621)	A	P	AQ	H/B	A
Technomyrmex butteli	A	?	AQ[i]	?	A
Aenictinae	1/2=50%				
**Aenictus javanus*	A	M	EQ	F	S
**Aenictus laeviceps*[a]	—	M	EQ	F	S
Dorylinae	0/1=0%				
**Dorylus laevigatus*	—	M	EQ	F	S
Leptanillinae	2/2=100%				
**Leptanilla* sp. 1 (FI97-556)	A	M?	EQ	F	S
**Leptanilla* sp. 2 (FI97-581)	A	M?	EQ	F	S
Myrmicinae	8/18=44%				
**Acanthomyrmex ferox*[b]	A	P	AQ	H/B	S
Cardiocondyla sp. (FI97-20)	A	M?	AQ?	?	S
Crematogaster sp. (FI97-627)	A	?	AQ?	?	A
Monomorium sp. 1 (FI97-16)	—	M–P	AQ[j]	H/B?	S
Monomorium sp. 2 (FI97-628)	A+B	M–P	AQ[j]	H/B?	S
**Myrmecina* sp. A[e]	—	P	EQ	F	S
Myrmicaria castanea	—	?	AQ	?	S
Pheidole plagiara	—	?	AQ	?	S
**Pheidole* sp. 1 (FI97-631)	—	M	AQ	H	S
Pheidole sp. 2 (FI97-80)	A	?	AQ	?	S
**Pheidole tandjongensis*	—	M	AQ	H	S
**Pheidologeton affinis*	A	M[k]	AQ	P	S
Pheidologeton sp. (FI97-31)	—	?	AQ?	?	S
**Pristomyrmex brevispinosus*[b]	—	M	AQ	H	S
Strumigenys koningsbergeri	A	M?	AQ	H?	S
Strumigenys sp. 2 (FI97-626)	A	M–P	AQ	H/B?	S
* *Tetramorium kheperra*	—	P	AQ	H/B	S
Tetramorium sp. (FI97-18)	—	P?	AQ	H/B	S
Pseudomyrmecinae	1/1=100%				
Tetraponera sp. (FI97-624)	A	?	AQ?	?	A
Ponerinae	5/14=36%				
**Amblyopone reclinata*[f]	A	P	G	B/F	S
* *Diacamma rugosum*[h]	—	M	G	B[i]	A/S
**Diacamma* sp. (FI97-273)[a]	—	M	G	B/F	S
**Gnamptogenys costata*[a]	—	M	AQ	H	S
**Gnamptogenys dammermani*	—	P[l]	AQ	H/B?	S
**Hypoponera* sp. (FI97-55)	—	M	AQ	H	S
* *Leptogenys diminuta*[g]	—	M	EQ[m]	F	S
**Leptogenys myops*[g]	A	M	EQ	F	S

(*continued*)

Table 1 (*continued*)

subfamily species (Bogor National Reserve Collection access number)	*Wolbachia* strain	social structure (M/P)	type of reproduction (G/GQ/EQ/AQ)	colony propagation (H/P/B/F)	nesting habits (A/S)
**Myopias emeryi*[d]	A	P	AQ	H/B	S
**Odontomachus simillimus*	—	M	AQ	H	S
**Odontomachus rixosus*	A	P	AQ	B/F	S
**Pachycondyla astuta*[c]	—	P	GQ	H/B/F	S
**Platythyrea* sp. (FI97-611)[b]	—	P	GQ	H/B/F	S
**Probolomyrmex dammermani*	A	M	AQ	H	S

[a] Collected from Ujung Kulon National Park.
[b] Collected from Mt Sarak (Bogor).
[c] Collected from Mt Gede (West Java).
[d] Collected from Padang (West Sumatra).
[e] Ito (1996).
[f] Ito (1993).
[g] Ito (1997).
[h] Fukumoto *et al.* (1989).
[i] *T. albipes* has alate queens and intercastes (Yamauchi *et al.* 1991). No data were available concerning the presence of intercastes in this species.
[j] An Australian *Monomorium* species of the *rothsteini* group has intercastes (Briese 1983). The presence of intercastes was not investigated in these species.
[k] Both polygynous and monogynous colonies were collected. Polygynous colonies have been proposed to be incipient colonies, implying a pleometrotic mode of colony foundation (Moffett 1988).
[l] This species is functionally monogynous, i.e. a single inseminated dealate queen coexists with a number of virgin dealate queens (F. Ito, unpublished data).
[m] Wilson (1958).

of the species of table 1 have not been studied in detail before, making it impossible to sort out possible confounding variables. Only more extensive data on well-studied species will allow firm conclusions in this respect.

Wolbachia infections also occur both in the normal alate queen species, as well as in the more atypical species where reproduction is performed by wingless ('ergatoid', EQ species in table 1) queens or mated workers ('gamergates', G and GQ species in table 1). Moreover, susceptibility to *Wolbachia* infection seems to be independent of nesting habits (table 1, $\chi^2 = 0.00$, d.f. $= 1$, $p > 0.05$).

4. DISCUSSION

The present study shows that *Wolbachia* is extremely common in Indonesian ants, with 50% of the species infected overall: the highest infection rate, we believe, ever reported in any animal group. The infection rate is between two and four times higher than that reported in a general screening of New World insect species (Werren *et al.* 1995*b*) and *Drosophila* (Bourtzis *et al.* 1996), and is also higher than the 35–40% positives reported in mites (Breeuwer & Jacobs 1996). When the infection rates among species with various social structures are compared, it becomes clear that this high incidence seems largely restricted to the dependently founding species class where 50% of the species are infected versus 13% for the independently founding species. In general, this unusually common occurrence could be due to (i) the use of different primer pairs perhaps also mapping to more conserved template regions, or (ii) facilitated invasion and/or particularly stable maintenance of *Wolbachia* in these social Hymenoptera. The latter idea is investigated below where we list some of the major factors that might influence the spread and maintenance of *Wolbachia* in hymenopteran societies. In this discussion and in the absence of any empirical data on the exact effects of the bacterium in ants, we will assume that cytoplasmic incompatibility is the predominant mode of action (which in insects is generally true (Werren 1997)), with feminization being ruled out (haploid queens would be sterile), and—as noted by Hurst & Peck (1996)—parthenogenesis induction being limited to the chalcidoid and cynipoid parasitoid wasps (Stouthamer 1997), the only hymenopteran taxa where the gamete duplication diploidization system typical of *Wolbachia* (Stouthamer 1997) does not lead to the production of diploid males instead of females (Cook 1993). Further evidence arguing against a general role in parthenogenesis induction is that whereas *Wolbachia* is very common in ants, female parthenogenesis (thelytoky) has been reported in only five out of *ca.* 8800 described species (Heinze & Tsuji 1995), and that those species are all *Wolbachia*-negative (T. Wenseleers, unpublished data).

(a) *Cytoplasmic incompatibility in social insects: additional 'society'-level selection*

Typically, a hymenopteran society can produce sexuals only after an initial ergonomic worker-producing phase. In such a system, cytoplasmic incompatibility would result in fitness deficits connected to both the individual and 'society' levels of organization of its host. At the 'society' level, incompatible matings would make queens experience a greater mortality during their colony founding stages because the phenotypic effect of lowering the primary sex ratio would make them very inefficient at producing female workers. In addition, any queens that did succeed in establishing a successful colony would tend to produce an above-average male-biased sexual brood

just as in solitary Hymenoptera (Saul 1961; Ryan & Saul 1968; Breeuwer & Werren 1990). Therefore, an incompatibility micro-organism like *Wolbachia* is expected to act more forcefully and spread more readily in a social host compared with a solitary one.

(b) *Population structure: high population viscosity associated with budding and fissioning*

Ant species reproducing by budding or fissioning are poor dispersers and are therefore usually characterized by a high population viscosity (e.g. Chapuisat *et al*. 1997). This might facilitate the spread of *Wolbachia* by reducing the effectively breeding population (effective population size), making stochastic processes allowing invasion of *Wolbachia* more likely (Turelli 1994; Turelli & Hoffmann 1991, 1995). The observed association between *Wolbachia* prevalence and presence of dependent colony founding is in agreement with this idea.

(c) *Evolutionary changes in* Wolbachia *and hosts*

A final possibility is that infection patterns are best explained not in terms of particular invasion criteria (as in (a) and (b) above), but mainly as a long-term outcome of selection for increased compatibility levels favoured by both the host and/or the parasite (Turelli 1994; Hurst & McVean 1996), a process that would ultimately lead to stochastic loss of infection. Evolutionary changes in the ant hosts might be expected by analogy with the similar risk of making a matched mating at the sex-determining locus (Cook 1993; Cook & Crozier 1995). In particular, dependent colony foundation (or polygyny) has been proposed to provide a way to evade fitness losses resulting from 'incompatible' matched matings (e.g. Ross & Fletcher 1985; Ross 1993). Multiple maternity might therefore be an alternative to multiple paternity (Zeh & Zeh 1996, 1997) as a strategy to avoid fitness losses resulting from cytoplasmic incompatibility. In this respect, selection for dependent founding or greater selection against sperm modification in the independently founding species class could be an alternative explanation for the observed association between *Wolbachia* infection and mode of colony foundation. The critical parameter that will need to be determined is whether the frequency of incompatible matings is high enough for the cost of a nuclear modifier to be offset by the benefit of reduced incompatibility.

It needs to be stressed that the three factors described above are not meant to be exhaustive in explaining the spread and maintenance of *Wolbachia* in social Hymenoptera. For example, the typical large and perennial nature of most ant societies makes them arguably highly susceptible to parasites (Boomsma & Ratnieks 1996). Most importantly, however, there is an urgent need for detailed data on the aetiology of *Wolbachia* in social Hymenoptera, so that the effects of incompatibility hypothesized in this and other papers (Hurst 1997) can either be refuted or confirmed. The present study at least shows that *Wolbachia* might be more common and important than previously thought in the social Hymenoptera, introducing yet another level of conflict in insect societies.

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